T/AU00/00408

REC'D 29 MAY 2000

4

Patent Office
Canberra

I, ANNA MAIJA EVERETT, ACTING TEAM LEADER EXAMINATION SUPPORT & SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 0204 for a patent by STEM CELL SCIENCES PTY LTD filed on 06 May 1999.

COMMONWEALTH OF AUSTRALIA
AUSTRALIA
PATENT OFFICE

WITNESS my hand this
Twenty-second day of May 2000

A. M. Everett.

ANNA MAIJA EVERETT
ACTING TEAM LEADER
EXAMINATION SUPPORT & SALES

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

P/00/009
Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: **Improved nuclear transfer and nuclear addition (1)**

The invention is described in the following statement:

IMPROVED NUCLEAR TRANSFER AND NUCLEAR ADDITION (1)

This invention relates to the generation of cells, embryos and animals by nuclear transfer or nuclear addition, including but not limited to the generation of transgenic animals.

Nuclear transfer or nuclear addition is the replacement or addition of the nucleus of one cell with that or to that of another. The ability to produce live offspring by nuclear transfer is an objective which has been sought for some time by animal breeders. The ability to produce cloned offspring in such a manner would enable the production of large numbers of identical offspring and the ability to genetically modify and/or select cell populations of the required genotype (e.g. sex or transgenic) prior to embryo reconstruction.

Whilst nuclear transfer has been described in some animals, the procedures used are often inefficient and have not yet been successfully applied to many species.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In a first aspect of the present invention there is provided a method of preparing a reconstituted cell which method includes

providing

a donor nucleus, and

a recipient cell;

optionally removing the nucleus from the recipient cell;

introducing the donor nucleus into the recipient cell to produce a reconstituted cell;

maintaining the reconstituted cell in a suitable medium for a period sufficient for cell recovery after introduction of the donor nucleus; and

subjecting the reconstituted cell to an activation step.

In a second aspect of the present invention there is provided a method of

generating an animal embryo which method includes

providing

a donor nucleus, and

a recipient cell;

optionally removing the nucleus from the recipient cell;

introducing the donor nucleus into the recipient cell to produce a reconstituted cell;

maintaining the reconstituted cell in a suitable medium for a period sufficient for cell recovery after introduction of the donor nucleus;

subjecting the reconstituted cell to an activation step; and

generating an animal embryo from the reconstituted cell.

Applicant has discovered that the number of viable embryos produced may be significantly increased by permitting the reconstituted cell to be maintained in a quiescent state prior to activation, and for a period sufficient to allow the cell to recover from the trauma of introduction of the donor nucleus.

Whilst applicant does not wish to be restricted by theory, it is postulated that the quiescent period permits the cell to return to a more normal state after which nuclear transfer activation may proceed more efficiently.

The reconstituted cell may be maintained in a suitable medium preferably for a period of approximately 3 to 8 hours, more preferably approximately 4.5 to 6 hours.

It is desirable, however, that the quiescent period end before any, or any substantive division, ensues.

Activation occurs during fertilisation when the penetrating sperm triggers the resumption of meiosis. Activation is characterised by calcium oscillation, release of cortical granules, extrusion of the second polar body, pronuclear formation and ultimately cleavage. The reconstituted cell may be treated with, but not limited to, for example, ethanol, calcium ionophore or electrical stimulation to induce activation. Activation is performed after the quiescent period.

In a preferred form of this aspect of the invention, the method includes the further step of

maintaining the activated reconstituted cell in a suitable medium for a period sufficient to allow the cell to recover a substantially normal shape.

Preferably the reconstituted cell is subsequently subjected to cell fusion.

The reconstituted cell may be subjected to a cell fusion/activation step. For example, where electrical pulses are utilised for cell fusion, the voltage may be selected to simultaneously initiate activation.

The reconstituted cell may also be subjected to simultaneous cell fusion/activation or a process of cell fusion followed later by activation.

The method of the present invention may include the further step of generating an animal from the animal embryo.

In a further aspect of the present invention there is provided a method of preparing a genetically modified reconstitution cell said method including

providing

a donor nucleus which has been genetically modified to eliminate or reduce an undesirable activity or to provide for or increase a desirable activity, and

a recipient cell;

optionally removing the nucleus from the recipient cell;

transferring the donor nucleus to the recipient cell to produce a genetically modified reconstituted cell;

maintaining the genetically modified reconstituted cell in a suitable medium for a period sufficient for cell recovery after introduction of the donor nucleus; and

subjecting the genetically modified reconstituted cell to an activation step.

In a still further aspect of the present invention there is provided a method of generating a transgenic animal embryo said method including

providing

a donor nucleus which has been genetically modified to eliminate or an undesirable activity or to provide for or increase a desirable activity, and

a recipient cell;

optionally removing the nucleus from the recipient cell;

transferring the donor nucleus to the recipient cell to produce a genetically modified reconstituted cell;

maintaining the genetically modified reconstituted cell in a suitable medium for a period sufficient for cell recovery after introduction of the donor nucleus;

subjecting the genetically modified reconstituted cell to an activation step; and

generating a transgenic animal embryo from said genetically modified reconstituted cell.

The reconstituted cell may be maintained in a suitable medium for a period of approximately 3 to 8 hours, preferably approximately 4.5 to 6 hours.

It is desirable, however, that the quiescent period end before any, or any substantive division, ensues.

Activation occurs during fertilisation when the penetrating sperm triggers the resumption of meiosis. Activation is characterised by calcium oscillation, release of cortical granules, extrusion of the second polar body, pronuclear formation and ultimately cleavage. The reconstituted cell may be treated with, but not limited to, for example, ethanol, calcium ionophore or electrical stimulation to induce activation. Activation is performed after the quiescent period.

In a preferred form of this aspect of the invention, the method includes the further step of

maintaining the activated reconstituted cell in a suitable medium for a period sufficient to allow the cell to recover a substantially normal shape.

Preferably the reconstituted cell is subsequently subjected to cell fusion.

The reconstituted cell may be subjected to a cell fusion/activation step. For example where electrical pulses are utilised to cell fusion the voltage may be selected to simultaneously initiate activation.

The method of the present invention may include the further step of generating a transgenic animal from said transgenic animal embryo.

The donor nucleus may be of any suitable type and from any suitable species. The donor nucleus may be contained in a karyoplast or cell. The donor nucleus may be of embryonic, embryonal tumor, foetal or adult origin. Donor nuclei may be prepared by removing the nucleus and a portion of the cytoplasm and plasma membrane surrounding it from early pre-implantation stage embryos (for example zygotes, 4- to 16- cell embryos) for example using microsurgery. When nuclei from more advanced embryonic cells are used the whole blastomere may be transferred to the recipient cytoplasm. Embryonic or foetal fibroblasts may be used. Embryonic stem (ES) cells [isolated from inner cell mass (ICM) cells, embryonic disc (ED) cells or primordial germ cells (PGC)] may be used. A cell line derived from an embryonal tumor may be used (eg. embryonal carcinoma (EC) or yolk sac tumor cells). Adult cells such as fibroblasts may also be used. In this case the whole cell may be fused to the recipient cytoplasm.

It is particularly preferred that the donor cells be at a particular stage in the cell cycle, for example G_0, G_1 or Sphase. Applicant has found that it is possible to isolate populations of cells which are enriched for cells at each stage in the cell cycle by sorting the cells on the basis of size, for example using FACS. This avoids the use of stains, which are toxic to the cells. Staining can be used on a sample of each size-sorted population to identify what stage in the cell cycle that population is at.

The recipient cell may be of any suitable type and from any suitable species. Recipient cells may be *in vivo* or *in vitro* produced oocytes or cytoplasts or may be prepared from *in vivo* or *in vitro* produced oocytes. Recipient cells may be oocytes or cytoplasts prepared from oocytes, for example arrested in the second metaphase of meiotic maturation (MII oocytes). Other sources of recipient

cells or cytoplasts include zygotes, fertilised oocytes, 2-cell blastomeres, and all lines produced from gonads.

Cytoplast preparation involves the removal of the nucleus in a process referred to as enucleation. The nucleus may be removed by microsurgery. This may involve the removal of pronuclei or metaphase plate and surrounding cytoplasm from zygotes or oocytes, for example by aspiration or embryo bisection. Such manipulation may follow incubation of the zygotes or oocytes in a microfilament inhibitor, for example cytochalasin B (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.), that relaxes the cytoskeleton and allows the removal of a portion of membrane enclosed cytoplasm containing the pronuclei or metaphase plate. Alternatively, nonphysical approaches such as inactivation of the chromosomes by UV (chemical) or laser irradiation may be used.

The donor nucleus may be transferred to the recipient cell by any suitable method. Such methods include, but are not limited to, microsurgical injection, and cell fusion for example mediated by electrical pulses (electrofusion), chemical reagents such as polyethyleneglycol or the use of inactivated virus such as Sendai virus.

Preferably the donor nucleus is introduced under the zona pellucida.

Cytoplast volume may be increased by fusing together zona pellucida free cytoplasts before, after or at approximately the same time as donor nucleus fusion.

An animal embryo may be generated from the reconstituted cell by any suitable method. Embryonic development may be initially *in vitro* and subsequently in a surrogate. Thus, the reconstituted cell may be initially cultured *in vitro* to produce an embryo and then the embryo may be transferred to a surrogate for subsequent development into an animal. *In vitro* culture of the reconstituted cells may be in any suitable medium.

The animal embryo or animal may be of any type, and includes bird, fish,

reptile and mammalian (including ungulate and primate) embryos including human embryos, and murine, bovine, ovine or porcine embryos. Preferably, the animal embryo is a porcine embryo, bovine embryo, murine embryo or human embryo.

In a preferred embodiment of this aspect of the present invention, the donor nucleus may be from an embryo that is itself the product of nuclear transfer or nuclear addition. This is known as serial nuclear transfer and/or addition.

Serial nuclear transfer and/or addition may improve the capacity of differentiated nuclei to direct normal development. Whilst applicant does not wish to be restricted by theory, serial nuclear transfer and/or addition is postulated to improve the developmental capacity of transplanted nuclei by allowing specific molecular components in the oocyte to assist in chromatin remodelling that is essential for nuclear reprogramming. Serial nuclear transfer and/or addition is not restricted to a singular event but may be initiated on more than one occasion to sequentially improve conditions for chromatin remodelling, nuclear reprogramming and embryonic development.

The donor nucleus and recipient cell which are used in the method of the present invention may be of any suitable origin. Preferably, they are of porcine, bovine, ovine, rodent, avian, fish, reptile, murine or human origin.

The method of the present invention may be used to generate transgenic animals. For example, a new gene may be expressed and/or an existing gene may be deleted in the transgenic animal. The addition of new genes is technically less demanding than the deletion of existing genes.

As used in this specification the term "transgenic", in relation to animals and all other species, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted or modified (either by

modifying the gene product or pattern of expression) is a transgenic animal for the purposes of this invention, as much as an animal to whose germ line an exogenous DNA sequence has been added.

The donor nucleus may be genetically modified by modifying, deleting or adding one or more genes. The gene(s) to be modified, deleted or added may be of any suitable type.

The process of modifying a gene may involve the introduction of one or more mutations in both copies of the target gene. Suitable cells may take up the mutation(s) and then be used to generate an animal. One copy of the gene may be disrupted in the cell and the resultant heterozygous animals bred with each other until one with both copies of the gene mutated is found. Alternatively, both copies of the gene may be modified *in vitro*.

To target an endogenous gene rather than introduce random mutations, a DNA construct (transgene) including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the target gene except for the introduction of the one or more mutations may, be used.

The targeting DNA may comprise a sequence in which the desired sequence modifications are flanked by DNA substantially isogenic with a corresponding target sequence in the genome to be modified. The substantially isogenic sequence is preferably at least about 97-98% identical with the corresponding target sequence (except for the desired sequence modifications), more preferably at least about 99.0-99.5% identical, most preferably about 99.6% to 99.9% identical. The targeting DNA and the target DNA preferably share stretches of DNA at least about 75 base pairs that are perfectly identical, more preferably at least about 150 base pairs that are perfectly identical, even more preferably at least about 500 base pairs that are perfectly identical. Accordingly, it is preferable to use targeting DNA derived from cells as closely related as possible to the cell being targeted; more preferably, the targeting DNA is derived from cells of the same haplotype as the cells being targeted. Most preferably, the targeting DNA is derived from cells of the same individual (or animal) as the cells being

targeted. Preferably, the targeting DNA sequence comprises at least about 100-200 base pairs of substantially isogenic DNA, more preferably at least about 300-1000 base pairs of substantially isogenic DNA, even more preferably at least 1000-15000 base pairs of substantially isogenic DNA.

As used herein, the term isogenic or substantially isogenic DNA refers to DNA having a sequence that is identical with or nearly identical with a reference DNA sequence. Indication that two sequences are isogenic is that they will hybridise with each other under the most stringent hybridisation conditions (see e.g., Sambrook, J., Fritsch, E.F., Maniatis, T., (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbour Laboratory Press, New York); and will not exhibit sequence polymorphism (i.e. they will not have different sites for cleavage by restriction endonucleases). The term "substantially isogenic" refers to DNA that is at least about 97-99% identical with the reference DNA sequence, and preferably at least about 99.5-99.9% identical with the reference DNA sequence and in certain cases 100% identical with the reference DNA sequence. Indications that two sequences are substantially isogenic is that they will still hybridise with each other under the most stringent conditions (see Sambrook, J., et al., 1989) and that they will only rarely exhibit restriction fragment length polymorphism (RFLP) or sequence polymorphism (relative to the number that would be statistically expected for sequences of their particular length which share at least about 97-98% sequence identity). In general, a targeting DNA sequence and a host cell sequence are compared over a window of at least about 75 consecutive nucleotides. DNA sequences compared between individuals of a highly inbred strain, such as the MHC inbred miniswine, are generally considered to be substantially isogenic even if detailed DNA sequence information is not available, if the sequence do not exhibit sequence polymorphisms by RFLP analysis.

Thus, the donor nucleus may be genetically modified by modifying an endogenous gene in the donor nucleus. The endogenous gene may be modified by introducing into said donor nucleus a DNA construct including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the endogenous gene and includes one or more mutations, such that there is

homologous recombination between the DNA construct and the endogenous gene.

The introduction of new genetic material and the subsequent selection of cells harbouring the desired targeted integration requires expansion and clonal selection of each founder transgenic cell. A limitation to applying this process in nuclear transplantation programs is the number of cell divisions which the transfected cell must undergo to provide sufficient material for molecular analysis of each transgenic colony and subsequent supply of nuclei for transfer. The great majority of cells suitable for *in vitro* genetic modification and subsequent nuclear transfer have limited *in vitro* propagation capacity. It is therefore desirable to utilise transfection and selection systems which generate and/or identify correctly targeted clones at high efficiency and with limited requirement for *in vitro* propagation.

A particularly efficient approach to selecting for correctly targeted clones is to use IRES gene trap targeting vectors, as described in Australian Patent 678234, the entire disclosure of which is incorporated herein by reference. The IRES gene trap targeting vector may be selected from IRES-neo, IRES-lacZ, (TAA_3) IRES-lacZ, (TAA_3) IRES-lacZ lox neo-tk lox, (TAG_3) IRES-lacZ/mclneo, SA lacZ-IRES neo, SA (TAA_3) IRES-nuclear lacZ, SA (TAA_3) IRES-nuclear lacZ lox Gprt lox, IRES-βgeo, (TAA_3) IRES-βgeo, SA IRES-βgeo SA Optimised IRES-β geo, IRES-nuclear βgeo, SA IRES-nuclear βgeo, SA (TAA_3) IRES-nuclear βgeo, SA Optimised IRES-nuclear βgeo, IRES-zeo, SA IRES-zeo, IRES-hph, SA IRES-hph, IRES-hph-tk, IRES-bsd, SA IRES-bsd, IRES-puro. IRES gene trap targeting vectors provide a significant enhancement in gene targeting efficiency by eliminating a large proportion of random integration events. IRES gene trap targeting vectors rely upon functional integration into an actively transcribed gene (such as the target gene) for expression of the selectable marker. Random integrations into non-transcribed regions of the genome are not selected.

In a preferred embodiment, it may be desirable to remove the selectable marker cassette from the targeted locus to eliminate expression of the eg.

antibiotic resistance gene. One approach is to flank the IRES selectable marker cassette with suitable DNA sequences which act as recombination sites following the addition of a suitable site-specific recombinase. One example of a suitable recombinase site is the lox site which is specific for the Cre recombinase protein. Another example of a suitable recombinase is the FLP/FRT recombinase system (O'Gorman, S., Fox, D.T., Wahl, G.M. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* 251(4999), 1351-5).

High efficiency gene targeting and selection has a significant advantage in that suitably stringent selection systems, such as the IRES gene trap targeting vectors, can eliminate the need for biochemical analysis of clonal cell lines. In this instance, individual nuclei from a pool of uncharacterised transgenic cells should generate offspring of the desired phenotype at a ratio equivalent to the selected pool. The elimination of clonal selection may be particularly useful where only limited *in vitro* propagation is desirable or possible. One such instance includes the culture of embryonic nuclei for nuclear transfer. Embryonic nuclei are more efficient than latter stage somatic cells for generating live born offspring by nuclear transfer. However, totipotential embryonic cells can not be cultured for extended periods for any other species than mice. Nuclear recycling of embryonic nuclei provides an opportunity to maintain, expand and genetically manipulate multipotential cells from animals *in vitro*.

The DNA constructs may be engineered in bacteria and then introduced into the cells. The transgenes may be introduced into the cells by any suitable method. Preferred methods include direct injection, electroporation, liposomes or calcium phosphate precipitation. Direct injection is the preferred method for embryonic cells while electroporation is more suitable for embryonic fibroblast and embryonic stem cell cultures.

Whilst applicant does not wish to be restricted by theory, it is thought that regions of substantially isogenic DNA either side of the mutation drag the transgene to the target site where it recombines and introduces the mutation. It is further thought that the main contributing factor for increasing the efficiency of

introducing a specific mutation in a given gene is the degree of similarity between the target DNA and the introduced DNA. Thus, it is preferred that the DNA is isogenic (genetically identical) not allogenic (genetically dissimilar) at the genetic locus that is to be targeted.

In a further aspect of the present invention there is provided a reconstituted animal cell or modified reconstituted animal cell produced by the methods of the present invention. Preferably the reconstituted animal cell or modified reconstituted animal cell is a porcine, murine, ovine, bovine, caprine or human cell.

In a further aspect of the present invention there is provided an animal embryo or transgenic animal embryo produced by the methods of the present invention. Preferably the animal embryo or transgenic animal embryo is a porcine, murine, ovine, bovine, caprine or human embryo.

In a still further aspect of the present invention there is provided an animal or transgenic animal produced by the methods of the present invention. Preferably the animal or transgenic animal is a porcine, murine, ovine, bovine, caprine or human animal.

The present invention will now be more fully described with reference to the accompanying Example. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLE

The effect of delaying activation in pig oocytes

Time delayed	No. of Oocytes	No. of Oocytes Fused (%)
1 – 2 hrs	131	57 (43.5)
4 – 6 hrs	104	63 (60.6)

Finally, it is to be understood that various alterations, modifications and/or [illegible] as outlined herein.

STEM CELL SCIENCES PTY LTD
By their Registered Patent Attorneys
Freehills Patent Attorneys

6 May 1999